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Inhibition of lymphocyte activation by catecholamines: evidence for a non-classical mechanism of catecholamine action

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SUMMARY

The effects of noradrenaline and other adrenergic agonists on lymphocyte activation were studied. Spleen and thymus cells from BALB/c mice were stimulated by mitogens and lymphocyte activation was monitored by measuring the incorporation of [methyl-^{3}H]thymidine into DNA. Noradrenaline, adrenaline, isoproterenol and dopamine all inhibited the activation of spleen and thymus cells by concanavalin A, a T-cell specific mitogen, and the activation of spleen cells by lipopolysaccharide, a T-independent B-cell mitogen. The various catecholamines were approximately equipotent, having IC_{50} of approximately 10 μM. α-adrenergic agonists (phenylephrine, clonidine) did not inhibit lymphocyte activation. Noradrenaline, adrenaline and isoproterenol also inhibited DNA synthesis in S49 T lymphoma cells. The effects of adrenergic receptor antagonists on lymphocyte function were also studied. The inhibition of lymphocyte activation by catecholamines could not be reversed by antagonists to β-adrenergic receptors (propranolol), α-adrenergic receptors (phentolamine), or dopaminergic receptors (haloperidol). Experiments with human peripheral blood leucocytes revealed that, as with murine cells, the β-adrenergic antagonists propranolol and nadalol did not affect the catecholamine-mediated inhibition of lymphocyte activation. Although lymphocytes contain β-adrenergic receptors that are coupled to adenylyl cyclase activity, catecholamines appear to inhibit murine lymphocyte activation by a mechanism that is independent of these or other classical adrenergic receptors.

INTRODUCTION

Communication between the nervous system and the immune system plays an important role in the regulation of responses to environmental stress and disease.[1,2] Lymphoid tissues are innervated by the sympathetic nervous system, suggesting that immune cells may be exposed to neurotransmitters in their reservoir organs.[3] Noradrenaline, the classical sympathetic neurotransmitter, inhibits lymphocyte activation in many systems[4–6] but the mechanisms by which this agent modulates lymphocyte function have not been elucidated. Because lymphocytes contain β-adrenergic receptors that are coupled to adenylyl cyclase,[7] and because cAMP mimics some of the effects of catecholamines on lymphocyte activation,[8] catecholamines have been thought to inhibit lymphocyte activation by a classical β-adrenergic receptor/adenylyl cyclase/cAMP-mediated pathway. However, the receptor that mediates the effects of catecholamines on lymphocyte activation has not been characterized. Here we report studies of the effects of adrenergic agonists and antagonists on lymphocyte activation in a defined serum- and protein-free culture system.[9]

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MATERIALS AND METHODS

Culture of spleen and thymus cells

Specific pathogen-free male BALB/c mice were purchased from Harlan (Indianapolis, IN) and maintained under barrier conditions. Mice were killed by cervical dislocation and their spleens or thymus glands were excised. At room temperature, the tissue was minced and gently pressed through a sterile Nytex nylon mesh (Tetko Inc., Elmsford, NY) with a sterile stainless steel laboratory spoon. The resulting single-cell suspension was washed twice with medium [RPMI-1640, with L-glutamine (Gibco, Grand Island, NY), supplemented with 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin]. Cells from four to five animals were pooled for each experiment. Cells (5×10^5 cells in 100 μl culture medium)

were placed in a 96-well flat-bottomed tissue culture plate and incubated at 37° under a humidified atmosphere of 95% air and 5% CO_2. Cell viability, as determined by trypan blue exclusion or fluorescein diacetate uptake, was > 85% at the start of culture and > 65% after 48 hr in culture; cell viability was not significantly affected by any of the agents tested. Human peripheral blood mononuclear cells obtained from heparinized venous blood were isolated by gradient centrifugation on Ficoll–Paque (Pharmacia, Piscataway, NJ) and cultured as described for murine cells, at a density of $2{\cdot}6 \times 10^5$ cells in a final volume of 200 μl.

Mitogenic stimulation of DNA synthesis

Cultures were stimulated with concanavalin A (Con A; Pharmacia, Piscataway, NJ) or lipopolysaccharide (LPS; phenol extract from *Escherichia coli* O55:B5; Sigma, St Louis, MO). These stimuli were used at concentrations (Con A, 0·2–1 μg/ml; LPS, 2–20 μg/ml, depending on the batch) that produced maximal stimulation of DNA synthesis.

Neurotransmitters or other pharmacological agents were added immediately before the mitogens. When antagonists were used, agents were added in the temporal order antagonist, agonist and mitogen. Adrenergic antagonists (D,L-propranolol, phentolamine, atenolol, yohimbine and haloperidol; Sigma) were added at a final concentration of 100 pM–100 μM. Approximately 5 min later, agonists (noradrenaline, adrenaline, isoproterenol, phenylephrine, clonidine, dopamine, carbachol and serotonin; Sigma) were added at a final concentration of 100 pM–100 μM. The agonists were dissolved in dilute HCl (final concentrations between 10^{-4} and 10^{-5} M). At the concentrations used, HCl by itself did not affect DNA synthesis by resting or mitogen-stimulated cells. After 44 hr of incubation, 1 μCi of [methyl-^{3}H]thymidine (6·7 Ci/mmol; DuPont, N. Billerica, MA) was added to each well. Four hours later the cells were harvested onto glass fibre filters (Grade 934AH; Whitaker, M.A. Bioproducts, Walkersville, MD) and washed 20 times with distilled water in a multiple sample harvester (Whitaker). The amount of [^{3}H]thymidine incorporated into DNA was measured by liquid scintillation counting and was expressed as c.p.m. [^{3}H]thymidine/culture well. Cells stimulated with mitogens typically incorporated 50 000–200 000 c.p.m./culture well. Unstimulated cells incorporated less than 3400 c.p.m./culture well; the various agents examined in these studies did not affect the incorporation of [^{3}H]thymidine into unstimulated cells. This basal rate of DNA synthesis was not subtracted from the rate seen in stimulated cells. Experiments were carried out in quintuplicates. DNA synthesis is presented either as mean ± SEM of quintuplicate determinations or as a percentage of the value seen in mitogen-stimulated cultures. Each experiment was repeated a minimum of two times. Statistical significance of the effects of catecholamines on DNA synthesis was determined by a random ANOVA followed by Dunnett's *t*-test.

Culture of S49 cells

S49 T-lymphoma cells were obtained from the University of California at San Francisco Cell Culture Facility. Cells were cultured in RPMI-1640 (with L-glutamine) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated horse serum (Gibco) at 37° in 95% air–5% CO_2; cells were subcultured every 3–4 days.

DNA synthesis in S49 cells

Cells (7×10^3 cells in 100 μl of medium) were placed in a 96-well flat-bottomed tissue culture plate. At the initiation of culture, cell viability as determined by trypan blue exclusion was > 98%. Adrenergic agonists or other pharmacological agents were added as described above. After 68 hr of incubation, 1 μCi of [^{3}H]thymidine was added to each well. Four hours later, the cells were harvested and the incorporation of [^{3}H]thymidine into DNA was determined as described above.

Adenylyl cyclase activity in permeabilized spleen cells

Adenylyl cyclase activity was assayed in spleen cells that had been permeabilized with saponin by the method of Rasenick & Kaplan.[10] Briefly, $1{\cdot}5 \times 10^8$ spleen cells were suspended in 3 ml saponin solution (140 mM potassium glutamate, pH 6·8, 2 mM ATP, 100 μg/ml saponin). After 160 seconds, 40 ml of 140 mM potassium glutamate was added to the cells and the cells were washed three times with Hanks' buffer (Gibco). Adenylyl cyclase activity was measured in a final assay volume of 0·4 ml. The reaction solution contained 0·5 mM ATP, 1 mM $MgCl_2$, 0·5 mM isobutyl methyl xanthene (IBMX) and $2–3 \times 10^6$ c.p.m. [α-^{32}P]ATP (610 Ci/mmol; ICN, Lisle, IL), in Hanks' buffer. Propranolol (10 μM) was added as indicated. After 5 min at room temperature, isoproterenol (50 μM) was added to activate adenylyl cyclase. The tubes were incubated for 15 min at 32°; reactions were terminated by boiling for 5 min, and [^{32}P]cAMP formed during the assay was isolated as described by Salomon *et al.*,[11] and quantified by liquid scintillation counting. Cell protein was measured by the method of Bradford.[12] Adenylyl cyclase activity is expressed as pmol cAMP formed/min/mg protein, mean ± SEM of triplicate determinations.

RESULTS

Effects of catecholamines on lymphocyte activation

In initial experiments, we studied the effects of catecholamines on the activation of T lymphocytes. Spleen cells and thymus cells from BALB/c mice were cultured in a serum- and protein-free medium and stimulated by Con A, a T-cell specific mitogen. Unstimulated cells had a low rate of replication and incorporated little [^{3}H]thymidine into DNA (< 3400 c.p.m.). Con A dramatically stimulated the incorporation of [^{3}H]thymidine into DNA of both spleen and thymus cells (Fig. 1a). Con A-stimulated [^{3}H]thymidine incorporation was inhibited by noradrenaline (approximately 50% inhibition at 10 μM, virtually complete inhibition at 100 μM). Adrenaline and isoproterenol also inhibited Con A-stimulated DNA synthesis both in spleen cells (Fig. 1b) and thymus cells (data not shown). The three catecholamines were approximately equipotent; at 10 μM all three agents inhibited [^{3}H]thymidine incorporation by about 50%. Noradrenaline also inhibited interleukin-2 (IL-2)-stimulated DNA synthesis by spleen cells (approximately 60% inhibition at 80 μM; data not shown). The inhibition of lymphocyte activation by catecholamines was not due to cell toxicity; cell viability at the end of the culture period ranged between 65% and 80% and was not affected by any of the agents used in these studies.

The spleen contains a mixed population of T and B cells. LPS, a T-cell independent B-cell mitogen, also stimulated the incorporation of [^{3}H]thymidine into DNA of spleen cells.

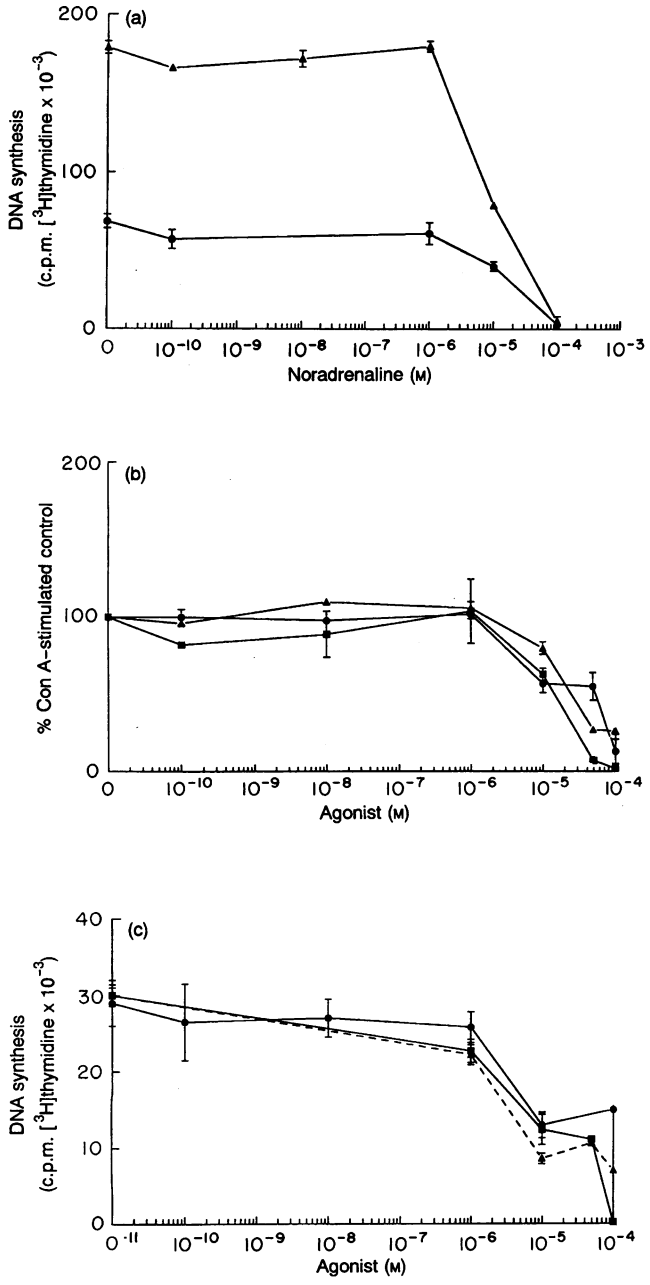

Figure 1. Inhibition of mitogen-stimulated DNA synthesis in spleen and thymus cells from BALB/c mice by catecholamines. (a) Spleen cells (▲) and thymus cells (●) were cultured with Con A (1 μg/ml) in the presence or absence of noradrenaline. DNA synthesis was measured by the incorporation of [^{3}H]thymidine into DNA as described in the text. (b) Spleen cells were cultured with Con A in the presence of various concentrations of noradrenaline (●), adrenaline (▲), or isoproterenol (■). DNA synthesis is expressed as a percentage of [^{3}H]thymidine incorporated into cells cultured with Con A alone. Controls for noradrenaline represent 119184 ± 32428 c.p.m., for adrenaline 109017 ± 11076 c.p.m., and for isoproterenol 90086 ± 1784 c.p.m. (c) Spleen cells were cultured with LPS (12 μg/ml) in the presence of various concentrations of noradrenaline (●), adrenaline (▲) or isoproterenol (■). DNA synthesis is expressed as a percentage of [^{3}H]thymidine incorporated into cells cultured with LPS alone (91976 ± 4402 c.p.m.). In all panels, data shown are means of quintuplicate determinations; SEM were smaller than the symbols.

Noradrenaline, adrenaline and isoproterenol all inhibited LPS-stimulated [^{3}H]thymidine incorporation into these cells (Fig. 1c). Again, these agents were approximately equipotent, having IC_{50} of the order of 10 μM. The effects of catecholamines on B-cell activation were probably not mediated by the T cells in these cultures, since noradrenaline also inhibited the LPS-stimulated incorporation of [^{3}H]thymidine into spleen cells from nude mice, which contain activation-incompetent T cells (data not shown). Dextran sulphate, a B-cell specific antigen, also increased [^{3}H]thymidine incorporation into spleen cells; noradrenaline inhibited the effect of dextran sulphate as well as the effect of LPS (40% inhibition at 50 μM noradrenaline, complete inhibition at 100 μM; data not shown). Thus, catecholamines inhibited the activation of both T cells and B cells by a variety of different agents.

The effects of other neurotransmitter receptor agonists on lymphocyte activation were also examined. Dopamine markedly inhibited both Con A-stimulated and LPS-stimulated [^{3}H]thymidine incorporation into spleen cells, whereas the α-adrenergic agonists phenylephrine and clonidine had no marked effect on the action of Con A or LPS (Table 1). Moreover, neither serotonin nor carbachol, in concentrations up to 100 μM, inhibited the action of either mitogen. The inhibition of mitogen-stimulated lymphocyte activation appears to be specific for catecholamines.

Table 1. Effects of neurotransmitter receptor agonists on mitogen-stimulated DNA synthesis in murine spleen cells

	Mitogen	
	Con A (1 μg/ml)	LPS (12 μg/ml)
Agonist	[^{3}H]thymidine incorporation (c.p.m.)	
None	91100 ± 2800	86500 ± 3000
Dopamine (10 μM)	77000 ± 2600*	64200 ± 2000*
Dopamine (100 μM)	10600 ± 2200*	6000 ± 100*
None	114200 ± 1600	77100 ± 1900
Phenylephrine (10 μM)	126300 ± 3700	72900 ± 1800
Phenylephrine (100 μM)	123000 ± 1200	73700 ± 1100
None	66800 ± 6500	88000 ± 1400
Clonidine (10 μM)	49200 ± 2800	84200 ± 6900
Clonidine (100 μM)	50600 ± 1200	ND
None	60700 ± 2400	119900 ± 1100
Carbachol (10 μM)	56200 ± 1500	127800 ± 1500
Carbachol (100 μM)	58900 ± 2200	125500 ± 2000
None	91100 ± 2800	86500 ± 3000
Serotonin (10 μM)	95900 ± 6600	70200 ± 2200
Serotonin (100 μM)	102800 ± 1500	91400 ± 4700

Spleen cells from BALB/c mice were cultured with Con A or LPS in the presence of the indicated agonists. The incorporation of [^{3}H]thymidine into DNA was measured as described in the text. Cells that were incubated in the absence of mitogens incorporated less than 3400 c.p.m. of [^{3}H]thymidine into DNA. Data shown represent mean ± SEM (n = 5).

* $P < 0.05$ compared to cells incubated with mitogen in the absence of an agonist.

ND, not determined.

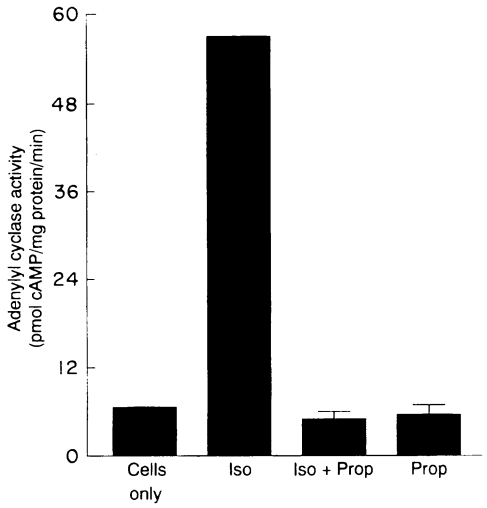

Figure 2. Adenylyl cyclase activity in spleen cells. Spleen cells were permeabilized with saponin and preincubated for 5 min at room temperature in adenylyl cyclase assay solution in the presence or absence of propranolol (10 μM; Prop). Isoproterenol (50 μM; Iso) was added as indicated and the incubation was continued for 15 min at 32°. Adenylyl cyclase activity was assayed as described in the text and is presented as pmol cAMP formed/min/mg of cellular protein.

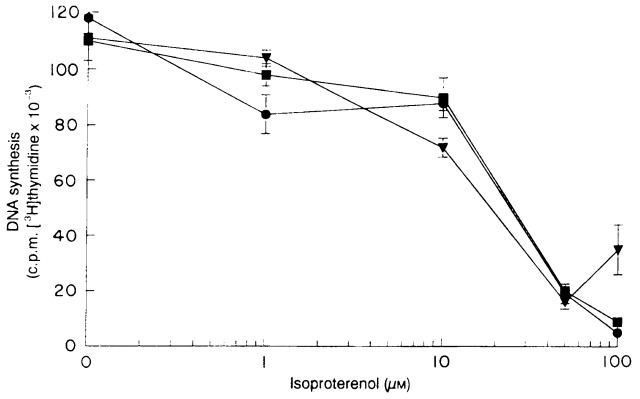

Figure 3. Failure of adrenergic antagonists to block inhibition of lymphocyte activation by catecholamines. Spleen cells were cultured with Con A (1 μg/ml) in the presence of various concentrations of isoproterenol and with propranolol (■, control; ●, 1 μM propranolol; ▼, 10 μM propranolol). DNA synthesis was measured as described in the text and is expressed as the mean of quintuplicate determinations; SEM were smaller than the symbols.

Catecholamines can interact with many different receptors, including members of the α-adrenergic, β-adrenergic and dopaminergic receptor families. Lymphocytes are known to contain all three receptor classes (β-adrenergic,[13] α-adrenergic,[14] dopaminergic[15]). β-adrenergic receptors in lymphocytes, as in many other tissues, are coupled to adenylyl cyclase activity. The spleen cells used in these experiments contained typical β-adrenergic receptor-coupled adenylyl cyclase activity; isoproterenol activated adenylyl cyclase activity in these cells, and propranolol, a β-adrenergic antagonist, blocked this effect of isoproterenol (Fig. 2). To identify the receptors that participated in the regulation of lymphocyte activation by catecholamines, we examined the effects of various receptor antagonists on catecholamine action. Propranolol did not block the inhibition of Con A-stimulated (Fig. 3) or LPS-stimulated [^{3}H]thymidine incorporation into spleen cells by isoproterenol. Moreover, the inhibition of lymphocyte activation by noradrenaline was not blocked by propranolol, by the

Table 2. Effect of adrenergic antagonists on catecholamine-induced inhibition of mitogen-stimulated DNA synthesis in murine spleen cells

	Con A (1 μg/ml)		LPS (2 μg/ml)	
	No agonist	Noradrenaline	No agonist	Noradrenaline
Antagonist	[^{3}H]thymidine incorporation (% of control)			
None (Exp. 1)	100	44 ± 1	100	58 ± 2
Propranolol (0·1 μM)	95 ± 2	50 ± 1	106 ± 4	65 ± 2
Propranolol (10 μM)	44 ± 2	32 ± 1	82 ± 2	58 ± 1
Phentolamine (0·1 μM)	94 ± 4	45 ± 2	116 ± 2	54 ± 1
Phentolamine (10 μM)	98 ± 2	49 ± 1	92 ± 2	52 ± 2
Propranolol (10μM) + phentolamine (10 μM)	ND	32 ± 2	ND	44 ± 4
None (Exp.2)	100	48 ± 5	100	22 ± 1
Haloperidol (50 μM)	121 ± 3	51 ± 3	101 ± 5	23 ± 2

Spleen cells from BALB/c mice were cultured with Con A or LPS in the presence or absence of noradrenaline (10 μM in Exp. 1; 50 μM in Exp. 2) and the indicated adrenergic or dopaminergic antagonists. The incorporation of [^{3}H]thymidine into DNA was measured as described in the text and is expressed as a percentage of the [^{3}H]thymidine incorporated into cells cultured with mitogen alone, mean ± SEM (n = 5).

ND, not determined.

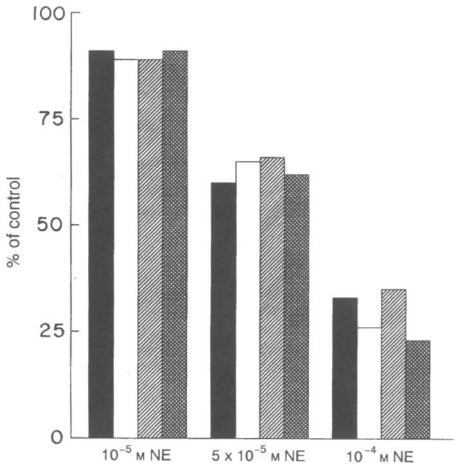

Figure 4. Failure of adrenergic antagonists to block inhibition of human lymphocyte activation by catechoamines. Human peripheral blood lymphocytes were cultured with Con A (1 μg/ml) and noradrenaline (NE) in the presence of the antagonists propranolol (□, 10^{-5} M; ▨, 10^{-6} M) or nadolol (▩, 10^{-6} M) or in the absence of antagonist (■). The incorporation of [^{3}H]thymidine into DNA was measured as described in the text. DNA synthesis is expressed as a percentage of [^{3}H]thymidine incorporated into cells cultured with Con A and noradrenaline (no treatment = 243 ± 13 c.p.m.; Con A = 21519 ± 1595). Data shown are means of quintuplicate determinations; SEM were < 10%.

α-adrenergic antagonist phenotolamine, or by the dopaminergic antagonist haloperidol (Table 2). To further substantiate that these effects were not mediated by adrenergic receptors, in other experiments neither the β-receptor antagonists atenolol or ICI 118 551 nor the α-receptor antagonist yohimbine blocked the inhibition of lymphocyte activation by noradrenaline (data not shown). Figure 4 shows that in a similar manner to the murine system, neither propranolol nor nadolol significantly altered noradrenaline responses in human peripheral blood lymphocytes.

Catecholamines also inhibited the replication of S49 cells, a cell line derived from a murine T-cell lymphoma.[16] As with normal lymphocytes, noradrenaline, adrenaline and isoproterenol were approximately equipotent in inhibiting S49 cell replication (IC_{50} 10 μM; Table 3) and the effects of catecholamines were not blocked by propranolol (Table 4). In addition, consistent with our observations reported above that T cells contain functioning β-adrenergic receptors as measured by the ability of propranolol to inhibit catecholamine-stimulated adenylyl cyclase activity, we showed that S49 cells also had functional β-adrenergic receptors. In S49 cells, Thy-1 gene expression is under β-adrenergic regulatory control and noradrenaline, isoproterenol and adrenaline inhibition of Thy-1 gene expression is readily reversed by propranolol.[17]

Table 3. Effects of adrenergic agonists on DNA synthesis by S49 T lymphoma cells

	DNA synthesis (% of control)		
Agonist (μM)	Noradrenaline	Adrenaline	Isoproterenol
1	86 ± 15	81 ± 12	81 ± 17
10	51 ± 25	60 ± 34	55 ± 33
100	1 ± 1	1 ± 1	1 ± 1

T lymphoma cells were cultured in the presence or absence of adrenergic agonists as indicated. The incorporation of [^{3}H]thymidine into DNA was measured as described in the text and is expressed as a percentage of the [^{3}H]thymidine incorporated into cells cultured in the absence of agonists. Data shown are mean ± SEM (n = 4).

Table 4. Effect of propranolol on DNA synthesis by S49 T lymphoma cells

	DNA synthesis [^{3}H]thymidine incorporation (c.p.m.)	
Noradrenaline (μM)	No antagonist	Propranolol (10 μM)
0	211 900 ± 8700	ND
5	125 100 ± 7400	131 700 ± 6600
10	84 500 ± 4200	51 100 ± 9200

T lymphoma cells were cultured in the presence of noradrenaline and propranolol as indicated. DNA synthesis is expressed as c.p.m. [^{3}H]thymidine incorporated per well, mean ± SEM (n = 5).
ND, not determined.

DISCUSSION

Previous studies have shown that catecholamines inhibit lymphocyte activation.[4–6] Since lymphocytes contain β-adrenergic receptors that are coupled to adenylyl cyclase, catecholamines raise cAMP levels in lymphocytes,[18] and cAMP analogues also inhibit lymphocyte activation,[9,19] catecholamines have been assumed to modulate lymphocyte function via a classical β-adrenergic receptor/adenylyl cyclase/cAMP-mediated mechanism. We have tested this hypothesis by examining the effects of a variety of adrenergic agonists and antagonists on lymphocyte activation. We carried out our experiments in a serum- and protein-free medium in order to avoid possible complications caused by undefined serum components. While our studies confirm the observations that catecholamines inhibit lymphocyte activation, they suggest that catecholamines affect lymphocyte activation through a mechanism that does not involve a classical adrenergic receptor. Such a mechanism could be cAMP-dependent or cAMP-independent.

A variety of adrenergic agonists, including adrenaline, noradrenaline, isoproterenol and dopamine, inhibits lymphocyte activation. β-adrenergic receptors in lymphocytes, like those in other tissues, have approximately 100-fold greater affinity for isoproterenol than for noradrenaline.[13] In contrast, in our studies these agonists were approximately equipotent in inhibiting murine lymphocyte activation. Moreover, although β-adrenergic antagonists blocked the activation of adenylyl cyclase by isoproterenol, the effects of catecholamines on lymphocyte activation were not inhibited by β-adrenergic, α-adrenergic or dopaminergic antagonists. Mitogens may alter the properties of the β-adrenergic receptors on lymphocytes.

Indeed, mitogens have been reported to synergize with β-adrenergic agonists in raising cAMP levels in lymphocytes.[20] However, S49 T lymphoma cells, which had not been treated with mitogens, exhibited similar patterns of adrenergic responsiveness as did normal, mitogen-treated lymphocytes. Alternately, catecholamines may affect lymphocyte function by activating an as yet uncharacterized adrenergic receptor. The description of a β_3-adrenergic receptor[21] emphasizes that there may be other, as yet unidentified, adrenergic receptors. (It is unlikely that the β_3-adrenergic receptor plays a role in the inhibition of lymphocyte activation by catecholamines, since ICI 118 551, an effective antagonist of this receptor,[21] did not antagonize the effects of noradrenaline on lymphocyte DNA synthesis.) Cells of the immune system have novel serotonin receptors[22] and cannabinoid receptors[23] and may also have novel adrenergic receptors. Finally, catecholamines may inhibit lymphocyte activation by a non-receptor-mediated mechanism. Autoxidation and the generation of reactive oxygen metabolites, and the binding of catecholamine oxidation products to proteins, are two possible non-receptor-mediated mechanisms by which catecholamines might modulate cell function.[24] Further work will be required to distinguish between these various alternatives and to define the mechanism of catecholamine action more completely.

Lymphoid organs are densely innervated by sympathetic nerves. The concentration of noradrenaline in the splenic parenchyma under resting conditions is about 1 μM;[3] this concentration is likely to be higher in the synapse-like spaces between sympathetic nerve terminals and lymphocytes, and to be further increased during periods of increased sympathetic activity. Thus, the concentrations of noradrenaline that inhibit lymphocyte activation *in vitro* are comparable to those to which lymphocytes presumably are exposed *in vivo*. Experiments with human peripheral blood lymphocytes revealed similar results for that system as well (Fig. 4), suggesting that the inability of lymphocytes to respond to propranolol may be of significance in human immunopharmacology.

Variations in sympathetic activity affect immune function.[5] By whatever mechanisms catecholamines affect lymphocyte activation, the effects of catecholamines that we have characterized are likely to be physiologically relevant and may underlie the modulation of the immune system by stress.

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